Mechanism-Based Inactivation of Caspases by the Apoptotic Suppressor p35†

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ABSTRACT: Caspases play a crucial role in the ability of animal cells to kill themselves by apoptosis. Caspase activity is regulated in vivo by members of three distinct protease inhibitor families, one of which—p35—has so far only been found in baculoviruses. P35 has previously been shown to rapidly form essentially irreversible complexes with its target caspases in a process that is accompanied by peptide bond cleavage. To determine the protease-inhibitory pathway utilized by this very selective protease inhibitor, we have analyzed the thermodynamic and kinetic stability of the protein. We show that the conformation of p35 is stabilized following cleavage within its reactive site loop. An inactive catalytic mutant of caspase 3 is bound by p35, but much less avidly than the wild-type enzyme, indicating that the protease catalytic nucleophile is required for stable complex formation. The inhibited protease is trapped as a covalent adduct, most likely with its catalytic Cys esterified to the carbonyl carbon of the scissile peptide bond. Together these data reveal that p35 is a mechanism-based inactivator that has adopted an inhibitory device reminiscent of the widely distributed serpin family, despite a complete lack of sequence or structural homology.

One of the most fundamental defensive responses to viral infection is death of the infected cells by apoptosis (*1*). Hence, animal viruses have developed several anti-apoptotic mechanisms to evade the defense response of the infected host. An extremely efficient way of accomplishing this is by direct inhibition of caspases, key proteolytic mediators in the initiation and execution of the apoptotic response (*2*, *3*). Two viral proteins, CrmA from cowpox viruses (*4*) and p35 from baculoviruses (*5*), can accomplish this to secure survival and replication within the infected host. CrmA and p35 are extremely potent caspase inhibitors (*6*), yet they have no structural or evolutionary relationship to each other (*7*, *8*). A fair amount is known about the inhibitory mechanism of CrmA, partly because it is a member of a broad family of well-characterized protease inhibitors, the serpins (*9*). In contrast p35 homologues are only known in baculoviruses. Surprisingly, there are no known host homologues, and much less is known of its inhibitory mechanism (*10*).

Evolution has developed several different solutions to the problem of how to inhibit proteases. Diverse protein scaffolds have been established to position surface loops that block the substrate-binding cleft of target proteases. Some inhibitors, such as the cystatins and the p41 I-chain fragment that inhibit some cysteine proteases (*11*), and the tissue inhibitors of metallo-proteases (*12*) utilize more than one loop to block the substrate cleft. However most inhibitors of serine proteases contain a single loop—the reactive site loop (RSL) that binds in a substrate like manner (*13*). Distinguishing these inhibitors from normal substrates is the extremely tight interaction and the very slow rate of hydrolysis (*14*). Even the cleaved (modified) inhibitor has equal efficiency, in a thermodynamic sense, though it may not be as kinetically active as the uncleaved (virgin) form (*15*). These "standard mechanism" inhibitors are tight binding, but reversible, resulting from a conformational constraint on the RSL. One family of protease inhibitors, the serpins, however, lacks this rigidity of the RSL. They act as mechanism-based inactivators (*16*), where cleavage of the RSL is followed by a dramatic conformational change altering the properties of the protein (*17*).

Interestingly, certain events that occur during the inhibition of caspases by p35 resemble the mechanism of serpins more than other inhibitor types. For example, p35 is cleaved during its reaction with caspases, and the resulting complex is so tight that it is difficult to measure the dissociation rate (*10*). The recently determined three-dimensional structure of virgin p35 demonstrates a mobile RSL (*7*), a feature shared with serpins even though there is otherwise no structural similarity. These features of p35 lead us to hypothesize that the protein is a mechanism-based inactivator and may share additional serpin-like features. We tested this hypothesis by examining a number of biophysical and biochemical properties of the virgin, protease-bound, and modified inhibitor. Our conclusions are supported by the recently determined crystal structure of the p35/caspase 8 complex (*50*).

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[1] Abbreviations: RSL, reactive site loop; Ac-DEVD-pNA, Acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; AFC, 7-amino-4-trifluoromethyl coumarin; GluSGP, *Streptomyces griseus* glutamyl peptidase; TUG, transverse urea gradient, GuHCl, guanidine hydrochloride; IAP, inhibitor of apoptosis protein; Z-VAD-FMK, benzoxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

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MATERIALS AND METHODS

Recombinant p35. The cDNA encoding *Autographa californica* nuclear polyhedrosis virus p35 plus a C-terminal His-6 purification tag was cloned into the expression vector pET21b(+) (Novagen, Madison, WI), expressed in *Escherichia coli* strain BL21(DE3)pLysS, and purified by a Ni-chelate affinity method (*10*).

Mutagenesis. Overlap PCR mutagenesis was employed to convert Cys 2 and 19 to Ala and Ser, respectively. The full sequence of each construct was checked by cycle-sequencing using ABI BigDye Terminator Kit on an ABI 377 sequencer (PE Applied Biosystems, Foster City, CA) to verify the success of the mutagenesis and the absence of any other mutations. The appropriate expression insert in was then transformed into *E. coli* strain BL21(DE3)pLysS, expressed and purified as for wild-type. The purity was judged to be greater than 95%, similar to that of the wild-type.

Proteases. Recombinant caspase 3 and a Cys285Ala catalytic mutant of caspase 3 lacking the pro-peptide (first 28 residues) were constructed and obtained following expression in *E. coli* (*18*). *Streptomyces griseus* glutamyl peptidase (GluSGP) was the gift of Henning Stennicke, The Burnham Institute (*19*); pancreatic elastase and *Staphylococcus Aureus* V8 glutamyl peptidase were obtained from Roche; trypsin and chymotrypsin from Sigma. Active-site concentrations of caspase 3 were determined (*20*) by titration with the irreversible caspase inhibitor Z-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK, where Z is benzoxycarbonyl; obtained from Enzyme System Products, Dublin, CA). The colorimetric caspase substrate Acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA) was purchased from Biomol, and the fluorogenic caspase substrate Ac-DEVD-AFC (where AFC is 7-amino-4-trifluoromethyl coumarin) was from Enzyme System Products (Dublin, CA). Assays using *p*NA substrates (maximal absorbance at 405 nm) were performed using a SpectraMAX 340 plate reader coupled with SOFTMAX software (Molecular Devices, Sunnyvale, CA). Assays using fluorogenic substrates were carried out on an *fmax* Fluorescence Plate Reader (Molecular Devices) at excitation wavelength 400 nm and emission wavelength 505 nm. All assays were thermostated at 37 °C.

Cleavage of p35. For screening proteases capable of cleaving p35, the purified recombinant protein (final concentration, 20μM; in 100 mM NaCl, 0.2 mM DTT, 1 mM EDTA, 50 mM Tris/HCl, pH 8.0) was incubated for 45 min at 37 °C with the respective protease at protease/p35 ratios of 1:100 to 1:10000. GluSGP was chosen to characterize the modified form of p35 (see below). The virgin protein was incubated under identical conditions, but in the absence of GluSGP. Both forms were subjected to ion-exchange chromatography (mono S column, Pharmacia) in 20 mM Bis-Tris Propane/HCl (Sigma), pH 6.5, using a 0−1 M NaCl gradient to obtain protease free protein.

Fluorescence Measurements and Guanidinium Hydrochloride (GuHCl) Unfolding. Fluorescence spectra of virgin and modified p35 (final concentration 750 nM, incubated 1 h at 25 °C in 20 mM Bis-Tris propane/HCl, 50 mM NaCl, in the presence of 0 or 6 M GuHCl, pH 6.5) were monitored on a Perkin-Elmer LS50B luminescence spectrometer coupled with the FL Win Lab software (Perkin-Elmer Corp., Northwalk, CT) using a 1 cm path-length cuvette (Helma). The excitation wavelength was 295 nm, and the emission was measured from 300 to 420 nm with a slit width of 5 nm for both excitation and emission. The stability of virgin and modified p35 was probed by incubating the proteins in the same buffer, in the presence of incremental increases in GuHCl (range 0−6 M), for 1 h at 25 °C. Then the fluorescence emission of the intrinsic tryptophans was measured at 350 nm.

Titration of p35 Forms and p35 Mutants with Caspase 3. For testing, the inhibitory activity and stoicheometry of inhibition different forms of p35 were incubated in final concentrations of 0−1 μM with 40 nM caspase 3 for 20 min at 37 °C in buffer [50 mM Hepes, 100 mM NaCl, 0.1%(w/v) CHAPS, 10% (w/v) sucrose, and 10 mM DTT, pH 7.4]. The measurement was started by adding 100 μM (final) Ac-DEVD-pNA substrate to the mixture (final volume, 100 μL).

SDS−PAGE. For monitoring the cleavage of p35 by various proteases 15% tricine SDS−PAGE was used (*21*). Otherwise a 8−18% linear acrylamide gradient PAGE with a 2-amino-2-methyl-1,3-propanediol/glycine/HCl system with SDS was used for resolving proteins (*22*). Normally, samples for SDS−PAGE were boiled in standard SDS sample buffer (84 mM 2-amino-2-methyl-1,3-propanediol/62 mM/glycine HCl, pH 9.1, 3.3% SDS) containing 16 mM DTT for 5 min and then loaded onto the stacking gel. Under certain circumstances, this procedure was replaced by other sample preparation conditions, as described in the relevant sections.

Mass Spectroscopy. Samples were separated by SDS−PAGE, stained with Coomassie blue and the bands of interest were digested at 37 °C for 4 h with 25 μg/mL trypsin dissolved in 50 mM ammonium bicarbonate. Samples were centrifuged 5000 rpm to pellet the gel slices, and 2 μL portions were spotted onto a sinapinic acid matrix for analysis by MALDI-TOF using a PE Biosystems Voyager DE-RP.

N-Terminal Sequencing of Protein Samples. For N-terminal sequencing, protein samples were resolved by SDS−PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA) by electroblotting (*23*). The membrane was stained with Coomassie blue and bands were excised and sequenced by Edman degradation on a 476A protein sequencer (Applied Biosystems, Foster City, CA).

RESULTS

Cleavage of the RSL by Nontarget Proteases. To investigate the properties of modified p35, we sought for a protease that cleaved within the RSL, preferably at the P1−P1′ scissile bond, without being inhibited. Therefore we incubated p35 with different amounts of trypsin, chymotrypsin, GluSGP, pancreatic elastase, and *S. aureus* V8 glutamyl peptidase. Although the most sensitive part of p35 to all of these proteases was within the RSL, GluSGP and elastase produced single cleavages that proved simple to analyze (Figure 1). N-Terminal sequencing located the cleavage site for both proteases within the RSL. While elastase cleaves p35 after Ile 93, the GluSGP cleavage site was after Asp 87, which is identical to the cleavage site produced by caspases during their inhibition (*24*).

Cleaved p35 Is Inactive and Shows Greater Stability against Denaturation. We used the RSL-modified material produced by GluSGP to test for inhibition of 20 nM caspase 3. While virgin p35 inhibits caspase 3 in an equimolar ratio,

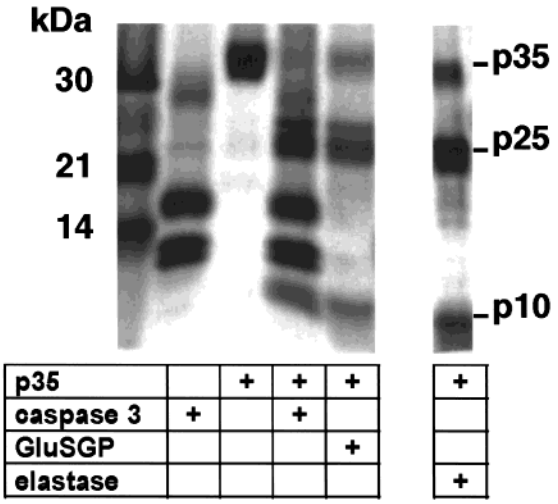

FIGURE 1: Cleavage of the RSL of p35. P35 is cleaved within the RSL resulting in a p25 and p10 fragment identical to the fragments seen upon caspase 3 inhibition. p35 (10 μM, final concentration) was incubated with either 20 μM caspase 3, 0.1 μM GluSGP, or 0.1 μM pancreatic elastase for 45 min at 37 °C. Samples were then subjected to SDS−PAGE in uniform 15% tricine gels (*21*).

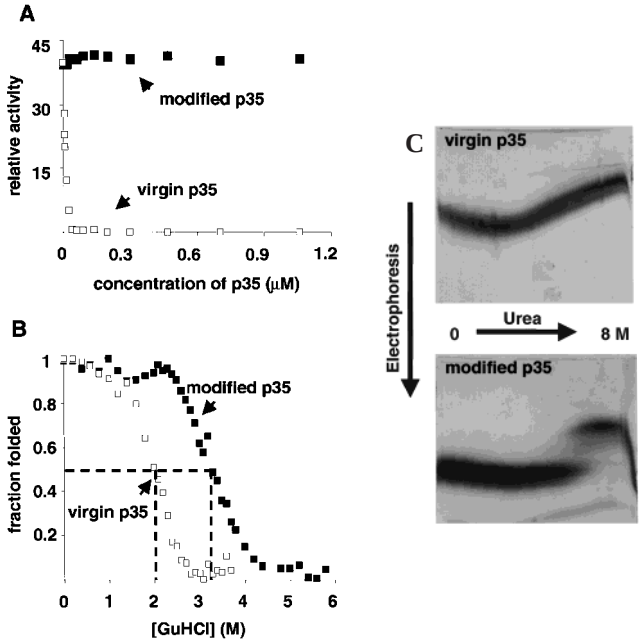

FIGURE 2: Characteristics of modified p35. (A) Inhibitory activity of virgin versus p35 modified by GluSGP. The modified form does not show any inhibition of caspase 3 up to 1.0 μM concentration, while the virgin form inhibits caspase 3 in an equimolar ratio. The indicated concentrations of p35 were preincubated with 40 nM caspase 3 for 1 h at 37 °C in caspase buffer. The measurement was started by adding Ac-DEVD-pNA to a final concentration of 200 μM. (B) GuHCl unfolding of virgin and modified p35. The latter shows greater stability than the virgin inhibitor with midpoints of unfolding (dashed lines) of 3.3 and 2.0 M GuHCl, respectively. Samples were incubated for 2 h at 25 °C in various concentrations of GuHCl before the intrinsic tryptophan fluorescence was measured (excitation/emission wavelength: 295/350 nm). (C) TUG gels of both p35 forms. Virgin p35 unfolds at al lower urea concentration than the modified form. The latter shows a discontinuous unfolding pattern, indicating slow unfolding in the time frame of electrophoresis. PAGE was run with the indicated urea gradient perpendicular to the electrophoresis direction at 4 °C for 2 h.

no inhibition could be seen with the modified inhibitor up to 1.0 μM, representing a several 100-fold molar excess (Figure 2A). We investigated the conformational stability of virgin p35 and its modified forms in GuHCl by measuring the intrinsic tryptophan-fluorescence of p35. Fluorescence emission spectra of the virgin and modified inhibitor both showed maxima at a wavelength around 350 nM (data not shown). Unfolding of p35 lead to a decrease in intrinsic fluorescence reaching the same absolute values for both virgin and modified form at high GuHCl concentrations. However, the modified inhibitor clearly unfolds at higher GuHCl concentration than the virgin inhibitor (Figure 2B). This implied that the conformational stability of the modified form was greater than the virgin form, and this differential was further investigated by transverse urea gradient (TUG)−PAGE. This method utilizes polyacrylamide gels with a urea gradient perpendicular to the direction of electrophoresis in otherwise nondenaturing conditions. Unfolding can be monitored by slower migration of the protein due to its larger hydrodynamic volume (*25*). As shown in Figure 2C, the modified form of p35 unfolds at higher urea concentration and is therefore more stable than the virgin form. The discontinuous unfolding pattern of modified p35 indicates a slow transition in the time scale of the electrophoresis (*25*). Interestingly, this may again point to a greater stability of the modified form, as observed in the case of lysozyme, where the wild-type protein and more stable mutants showed a discontinuous unfolding while less stable mutants showed continuous unfolding transitions (*26*).

P35 Binds to Processed Active-Site Mutants of Caspase 3, but Not the Unprocessed Zymogen. The P1 Asp of p35 had previously been shown to be essential for inhibition, pointing out the important role of substrate-like recognition in the inhibitory mechanism (*24*). However, previous studies had not demonstrated whether either the catalytic mechanism, proteolysis of the RSL, or both was required. Therefore, we tested whether the catalytic mechanism is crucial for binding of p35 to caspase 3 by utilizing a Cys285Ala catalytic mutant of the protease (*27*). Recombinant wild-type caspase 3 is translated as an inactive zymogen, which undergoes autoprocessing in the interchain linker during expression to yield the active two chain enzyme with a properly formed and accessible active site. As a consequence, the Cys285Ala active-site mutant is obtained in the zymogen form since it cannot autoactivate (*27*). However, an active conformation devoid of catalytic activity can readily be generated by allowing processing of the interchain linker by catalytic amounts of the physiologic caspase activator, granzyme B (*27*). To test the binding of p35 to the zymogen conformation or the processed catalytic mutant, size exclusion and gel shift (not shown) experiments were performed. The processed catalytic mutant was able to form a complex with p35, but the unprocessed catalytic mutant, in the zymogen conformation, was unable to form a detectable complex (Figure 3). Binding to the processed form in this experiment suggests an affinity at least in the micromolar range for the inhibitor-protease interaction in the absence of the catalytic Cys.

To assess the interaction between p35 and the processed catalytic mutant of caspase 3 we performed competition experiments in which the inhibitor (1 nM) was preincubated with different amounts of caspase 3 catalytic mutant (10 nM to 1 μM) before active caspase 3 was added. Inhibition was then analyzed by progress curves (not shown). The processed

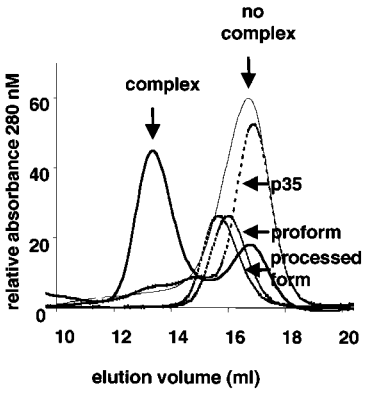

FIGURE 3: Binding of p35 to processed active site mutants of caspase 3. Gel filtration experiments with p35, caspase 3 Cys285Ala (proform) and granzyme B processed caspase 3 Cys285Ala (processed form). The elution profiles of the single components alone are shown as dashed lines. To investigate complex formation, p35 and either processed or unprocessed active site mutant of caspase 3 were incubated for 10 min at 25 °C at final concentrations of 20 μM (p35) and 10 μM (caspase mutants). The elution profile of p35 with unprocessed caspase 3 active-site mutant (thin line) encompasses the single components, indicating no binding between them. The processed form however is able to form a complex with p35 (thick line) with an apparent molecular mass of ~125 kDa (expected size 130 kDa).

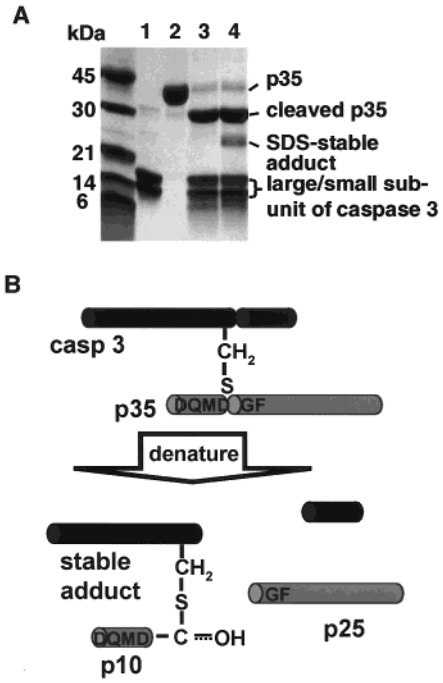

FIGURE 4: P35 forms an SDS-stable adduct with caspase 3. (A) SDS−PAGE of the p35 caspase 3 complex. The complex of p35 and caspase 3 was formed by incubating the components (final concentrations: caspase 3 20μM, p35 10 μM, 0.6 mM DTT) for 10 min at 25 °C. Samples were then subjected to SDS−PAGE after different pretreatments. Lanes 1−3, caspase 3, p35 and the complex of p35 and caspase 3 boiled for 2 min in sample buffer (pH 9.1) in the presence of 16 mM DTT (standard treatment); lane 4, pretreatment of the complex by boiling for 2 min at pH 6.5 without adding additional DTT resulted in an new band with an apparent molecular weight of 24 kDa. (B) Schematic representation of the complex formation between caspase 3 and p35. Caspase 3 consists of a large and a small subunit. The catalytic cysteine in the large subunit attacks p35 in the RSL, following $DQMD_{87}$. This results in cleavage of p35, giving an adduct stable to denaturation due to a thiol ester linkage between the N-terminal fragment of p35 (p10) and the large subunit of caspase 3, and dissociation of the small caspase subunit and the p25 fragment of p35 (starting with the sequence GF).

catalytic mutant was able to slightly decrease the overall k_{obs} of inhibition, but mutant concentrations up to 1 μM were not able to affect the final inhibition rates. This indicates a far less effective mode of binding in the absence of the catalytic Cys.

Identification of an SDS−Stable Adduct between p35 and Caspase-3. Mechanism-based inactivators generally form covalent adducts to the enzyme's catalytic residues, and in the case of the p35 caspase complex, this may manifest as a thiol ester between the catalytic cysteine and the carbonyl carbon of the scissile bond of the inhibitor. Because thiol esters are sensitive to nucleophilic attack by common buffers used for SDS−PAGE analysis, we employed modified treatment conditions in order to trap a putative ester between p35 and caspase 3. The expected mass of an adduct between the catalytic cysteine of caspase 3 and the carbonyl carbon of the scissile bond of p35 should encompass the protease large chain (17 kDa) and the N-terminal fragment of p35 (10 kDa). Boiling the complex for 2 min in the presence of 3% SDS at pH 6.5, without further addition of DTT (final concentration, 0.66 mM, resulting from the reducing conditions required to activate caspase prior to complex formation), followed by SDS−PAGE under our usual conditions revealed a new band migrating with an apparent size of 24 kDa (Figure 4A). This band is consistent with the predicted size of the cross-linked chains (Figure 4B). Immunoblotting showed a reaction of this band with both caspase 3 and p35 antisera (data not shown). To identify which chain of caspase 3 and which fragment of p35 were contained in this band, we excised the band and performed in-gel tryptic digestion followed by MALDI-MS. The mass spectrum shown in Table 1 provides clear evidence that the new band is composed of the large subunit of caspase 3 and the N-terminal fragment of p35, which was expected for an acyl−enzyme complex (Figure 4B). An anticipated fragment containing a covalent linkage between the two proteins could not be recovered, which is not unexpected given the relatively harsh conditions required for sample preparation prior to tryptic digestion, and the lability of thiol esters.

Table 1: Mass Analysis of Peptides Derived from the SDS-Stable p35/Caspase 3 Adduct

observed mass	expected mass	relative intensity[a] (%)	sequence	origin
758.49	758.43	29	KNNNLR	p10 of p35
797.55	797.43	100	ITNFFR	ls[b]/casp 3
878.49	878.50	35	ELVYINK	p10 of p35
925.53	925.52	22	KITNFR	ls/casp 3
1020.59	1020.56	93	LFIIQACR[c]	ls/casp 3
1118.49	1118.54	52	SGTDVDAANLR	ls/casp 3
1278.96	1278.60	96	VDEQFDQLER	p10 of p35
1494.14	1493.72	87	SKVDEQFDQLER	p10 of p35

[a] Intensity of 100% corresponds to 62 663 counts/second. [b] ls, large subunit of caspase 3. [c] Cysteine modified with iodacetamide.

Nature of the SDS-Stable Complex. Since thiol esters have characteristic sensitivities to pH and nucleophilic attack, we investigated the nature of the observed adduct by treating the complex under different conditions prior SDS−PAGE. SDS−PAGE under the same conditions as described above, but in the presence of 16 mM DTT, or boiling for 10 min, abolished the adduct (Figure 5A). The same result was

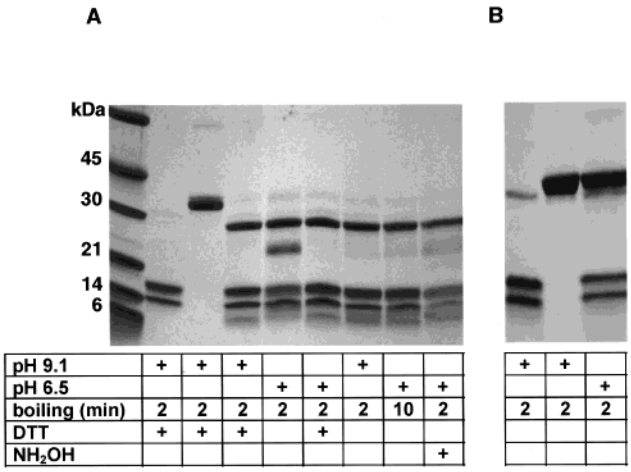

FIGURE 5: Properties of the SDS-stable adduct. (A) The complex of p35 and caspase 3 was subjected to different preteatments prior to SDS−PAGE as indicated in the table below the gel. The first lane is sizing standards, and the second and third lanes (as well as the first and second lanes in panel B) are controls of caspase 3 and p35, respectively. Samples were boiled for 2 or 10 min at pH 9.1 or 6.5 in the presence or absence of 16 mM DTT or 1 M NH_2OH. Alkaline pH, DTT or boiling for 10 min abrogated the SDS-stable adduct. The presence of 1 M NH_2OH strongly diminished the amount of adduct. (B) A complex of p35 and processed active site mutant of caspase 3 did not yield an SDS-stable adduct visible by SDS−PAGE, indicating the importance of the catalytic Cys.

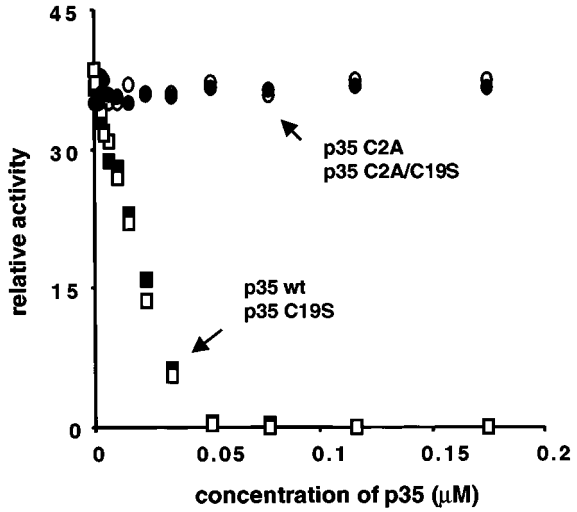

FIGURE 6: p35 mutation Cys2Ala converts p35 to a caspase substrate. Titration of p35 double mutant Cys2Ala/Cys19Ser (●), or p35 single mutants Cys19Ser (■) or Cys2Ala (○), or wild-type p35 (□) with caspase 3 shows that mutation of Cys2 to Ala abolishes inhibitory activity. The indicated concentrations of p35 were preincubated with 40 nM caspase 3 for 30 min at 37 °C in caspase buffer. Residual protease activity was measured after addition of Ac-DEVD-pNA to a final concentration of 200 μM.

obtained when the sample buffer pH was raised to 9.1. Addition of 1 M NH_2OH at pH 6.5 significantly diminished, but did not abrogate, the complex-band. If the adduct is indeed a thiol ester it should not form with a catalytic Cys285Ala mutant of caspase 3. Hence, pretreatment of this complex under the conditions described above did not result in any bands resembling an adduct between the large subunit of caspase 3 and p35 (Figure 5B).

Though the observed sensitivity to nucleophiles of the SDS stable adduct is characteristic of a thiol ester, there is a formal possibility that the adduct could form as a disulfide between p35 and the catalytic thiol of caspase 3. To test this, we created the double mutant p35 Cys2Ala and Cys19Ser to delete the Cys residues in the p10 fragment of p35. Surprisingly, this resulted in complete loss of inhibitory activity, and this effect was traced to Cys2, since the single Cys19Ser mutant still remained a potent caspase 3 inhibitor, and the single mutation Cys2Ala abrogated inhibitory activity (Figure 6). N-Terminal sequence analysis revealed an unblocked N-terminus in the p35 Cys2Ala mutant starting at position 2, while the active forms of p35 (wild-type and Cys19Ser) could not be sequenced indicating a modified/acetylated terminus. This indicates that the active forms of p35 have a blocked Met as the first residue, but mutation of Cys 2 results in processing of the protein, presumably by methionine aminopeptidase.

DISCUSSION

The essence of a mechanism-based inactivator is to use the catalytic properties of an enzyme to obtain inhibition (*28*). The inhibitor is initially treated as a substrate, but somewhere along the reaction coordinate an intermediate is stabilized, and the enzyme is trapped. In proteases, this usually takes place by a covalent modification to the catalytic nucleophile (Ser or Cys, depending on the protease type) or its assisting general base (His) and is a design feature modeled into many synthetic inhibitors (*29*). Concomitantly with inactivation of the enzyme, the inhibitor itself is inactivated. Thus, these type of inhibitors are also called "Trojan horse" or "suicide" inhibitors. It is unusual for proteins to act as mechanism-based inactivators, and most types avoid the catalytic machinery by simply blocking the substrate cleft in a more-or-less lock and key type of interaction (*12*, *14*).

The cleavage of p35 upon caspase inhibition distinguishes it from all natural inhibitors with the exception of the α-macroglobulins and the serpins. The α-macroglobulins constitute a unique inhibitor family that acts by trapping almost all endopeptidases, irrespective of catalytic mechanism (*30*, *31*). The protease activity is not formally inhibited, but the active site is shielded within the core of the large α-macroglobulin structure. Activity against small (synthetic) substrates is retained, but steric clashes prevent the access of large (protein) substrates to the trapped protease. This is not the case with p35, since all activities are abolished upon binding of a target protease, including those against small synthetic substrates (*10*).

The proteolytic modification of p35 is more like that of the serpins, where a partial refolding of the inhibitor takes place upon cleavage of the RSL (*32*). We were able to obtain modified p35 by digesting the inhibitor with GluSGP, which cleaved at Asp87, the P1 residue utilized during inhibition of caspases. The cleavage led to complete loss in inhibitory activity of p35. This is in contrast to standard type inhibition, where modified forms still can inhibit their target proteases, and an equilibrium between the modified and virgin form is reached (*15*). This behavior originates from the relative rigidity of the RSLs present in standard type inhibitors. Our observation that modified p35 is inactive is in accord with the crystal structure of p35, which shows an extremely exposed and flexible RSL (*7*). Serpins, which also possess a flexible RSL, show a complete loss of activity upon

cleavage. These inhibitors undergo a large conformational rearrangement, where the chain N-terminal to the cleavage site completes a five-stranded β-sheet leading to conformational stabilization of the modified inhibitor (*33*). We observed both by GuHCl unfolding and TUG−PAGE a greater stability of p35 against the denaturant. This is in contrast to standard mechanism serine protease inhibitors (and indeed almost all other proteins), where proteolysis leads to a decrease in stability (*34*), but shows similarity to the behavior of serpins.

Cleavage of p35 shifted the 50% unfolding point from 2.0 M GuHCl to 3.3 M, which is slightly smaller than the magnitude of the transition reported for the serpin plasminogen activator inhibitor 1, where the virgin form unfolds at GuHCl concentrations around 2 M, while the modified forms are stable up to 4 M (*35*). In TUG−PAGE, modified p35 unfolded around 6 M urea whereas serpins, including the caspase inhibitor CrmA, show no transition up to 8 M Urea (*36*, *37*). Thus, p35 shares with serpins the unusual conformational stabilization following RSL cleavage, with the virgin forms being among the least stable of folded proteins (*37*). However, the magnitude of the cleavage-driven conformational stabilization is considerably less than in serpins. The observed stabilization of p35 could result from a conformational change upon cleavage, as with serpins, but it must have a different structural basis as the structure of p35 bears no similarity to the serpin fold.

Significantly, the nonprocessed ("pro-") form of caspase 3 Cys285Ala was unable to interact with p35, suggesting that the substrate binding sites of the pro-enzyme are unavailable or not properly formed. In contrast, a processed catalytic mutant of caspase 3 was able to bind p35, but the affinity was only in the micromolar range. This contrasts with catalytically active caspases, which typically interact so tightly with p35 that their affinities are almost impossible to measure (*10*). Thus, the catalytic Cys appears crucial for the tight inhibition of caspases by the inhibitor. Interestingly, blocking the substrate binding cleft with a tetrapeptide fluoromethyl ketone completely abrogates binding of caspase 3 to p35 (*10*). The fact that the processed catalytic mutant of caspase 3 can bind to p35 indicates that additional interactions, besides the catalytic Cys, but within the general area of the substrate binding cleft, must contribute to protease recognition and eventual complex formation. Indeed, we propose that these interactions account for the high selectivity of p35 toward members of the caspase family (*10*, *24*, *38*). An influence of contacts beyond the catalytic site has also been suggested for complexes of serpins with catalytic mutants of serine proteases, and has been proposed to play a role in the large differences in the affinity (ranging from 10 nM to over 10 μM) for various serpins to these mutant proteases (*39*).

The characteristics of the SDS-stable adduct formed between p35 and caspase 3 are consistent with a thiol ester, which would be equivalent to the acyl enzyme intermediate in hydrolysis of a substrate. The most convincing evidence for this comes from the inability to form the adduct when the catalytic Cys of caspase 3 is mutated to Ala. There is also a formal possibility that this adduct represents a disulfide between the caspase catalytic Cys and Cys2 of p35, especially since mutation of Cys2 in p35 abrogated SDS-stable complex formation and inhibition. However, sensitivity to NH_2OH, elevated pH, and prolonged boiling are more diagnostic for thiol esters than for disulfides. The adduct was also destabilized in the presence of 16 mM DTT, but only following boiling. Though DTT reduces disulfides, it also cleaves thiol esters (*40*). Therefore, we conclude that the SDS-stable adduct is most likely a thiol ester between the catalytic Cys285 in caspase 3, and the carbonyl moiety of Asp87 in p35 (Figure 4B), and that the inhibitory complex is stabilized in this manner. Indeed, while this manuscript was in review, the crystal structure of the complex between p35 and caspase 8 was published, supporting our conclusions regarding the nature of the complex between p35 and caspase 3. In the crystal structure, the caspase 8 catalytic Cys is linked by a thiol ester to the scissile bond of p35 (*50*). The inhibitor has undergone a profound conformational change, as revealed by our unfolding data, with the repositioning of the N-terminus into the active site of the caspase (*50*). The structure explains the absolute requirement of p35 for Cys2, since this residue seems to form a H-bond with the general base His237 of caspase 8 that may distort it away from its partner, the catalytic Cys285.

An equivalent formation of SDS-stable adducts between the catalytic Ser of serine proteases and a scissile bond in the inhibitor is a hallmark of serpins (*41*, *42*). A covalent linkage of serpins with target cysteine proteases, as opposed to serine proteases, is implied in the well-characterized serpin mechanism (*17*). However, only one example of a covalent adduct between a cysteine protease and a serpin, a thiol ester between cathepsin L and squamous cell carcinoma antigen 1, has been proposed to date (*43*).

With the exception of the serpins and p35, all other natural active-site-directed protease inhibitors react noncovalently with their targets. Thus, p35 and the serpins share several characteristics in common: conformation stabilization by proteolysis, inactivation by proteolysis at the scissile bond, SDS-stable adduct formation, and almost complete irreversibility. On these grounds, we propose that they share the same mode of action, which would be due to mechanism-based inactivation. However, the reaction pathway that results in the inhibition must be distinct, since serpins show no structural homology with p35. For example, the N-terminus of serpins has no known role in inhibition (*8*), but the activity loss of the Cys2Ala mutant implies a critical role for the N-terminal region of p35.

P35 is now the second example of a protein fold that utilizes mechanism-based inactivation of its target proteases, and this has an advantage over other inhibitory strategies. Caspases share a common catalytic machinery, but their substrate binding surfaces are greatly varied, accounting for their individual substrate preferences (*44*). The other two caspase inhibitors, CrmA and IAP family members are much more limited in their specificity [CrmA targets caspases 1, 8, and 10 (*45*); XIAP targets caspases 3, 7, and 9 (*46*)]. Their limited reactivity is due to specific binding requirements of the caspase substrate cleft and steric restrictions on the RSLs of the inhibitors (*47*−*49*). But p35 has an extremely large and flexible RSL that allows the inhibitor to adapt to multiple caspases. The broad reactivity, combined with its ability to trap caspases once they have cleaved the loop makes p35 an ideal tool for the virus to evade the apoptotic response of infected host cells.

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